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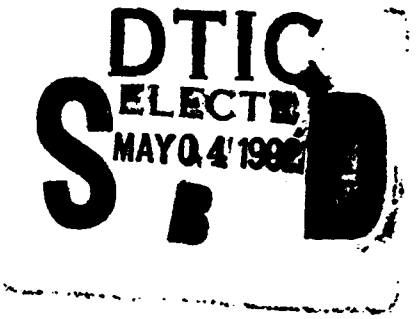
NEW APPROACHES TO HEPATITIS A VACCINE DEVELOPMENT

ANNUAL REPORT

Stanley M. Lemon, M.D.



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Hepatitis A virus (HAV) has historically been an important cause of morbidity among U.S. soldiers in the field. Work under this contract is directed at the development of a safe, inexpensive and effective hepatitis A vaccine for use in military personnel. Two main research approaches are under investigation. First, we are characterizing neutralization escape mutants of HAV and examining synthetic oligopeptides representing the suspected surface structures of HAV. Octapeptides have been synthesized on polyethelene pins and probed with polyclonal and monoclonal antibodies in an effort to identify antigenic and potentially immunogenic sequences. We have also determined the ability of poliovirus-HAV chimeric viruses to elicit neutralizing antibodies to HAV. The second approach involves the construction of an infectious cDNA construct derived from a virulent but cell culture-adapted variant of HAV. Genetic manipulation of such a construct will allow alternative approaches to the development of attenuated vaccine candidates. A third aim is to utilize PCR-related technology for identification of specific strains of HAV

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FOREWORD

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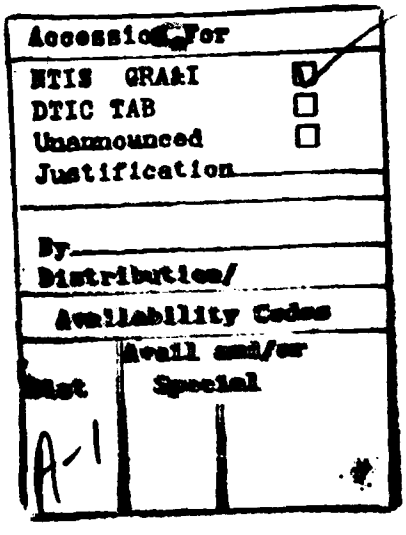
The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.



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ABSTRACT

Hepatitis A virus (HAV) has historically been an important cause of morbidity among U.S. soldiers in the field. Work under this contract is directed at the development of a safe, inexpensive and effective hepatitis A vaccine for use in military personnel. As in the preceding years, two main research approaches are under investigation. First, the primary goals of this contract are the characterization of neutralization epitopes on the surface of the HAV capsid and an examination of the antigenicity of synthetic oligopeptides representing the suspected surface structures of HAV. Octapeptides have been synthesized on polyethelene pins and probed with polyclonal and monoclonal antibodies in an effort to identify antigenic and potentially immunogenic sequences. These studies have recently defined a linear antigenic site on the virus capsid which is reactive with a murine neutralizing monoclonal antibody. As a subsidiary aim, we continue the investigation of poliovirus-HAV chimeric viruses which have been engineered to express potential HAV neutralization determinants. The second major approach involves basic studies related to genetic mechanisms of attenuation of HAV. This work has involved construction of a genomic length cDNA construct derived from a cell culture-adapted variant of HAV, and the characterization of mutations which enhance the growth of hepatitis A in cell culture, as these mutations are associated with attenuation of the virus. These studies now focus on mutations involving the 5' nontranslated region (5' NTR) of HAV. The 5' NTR plays a key role in determining the attenuation phenotype of the Sabin poliovirus vaccine strains and we have shown recently that mutations in the 5'NTR of HAV are critical for the enhanced growth of cell culture-adapted variants. We anticipate that these studies will lead to new approaches to the construction of genetically engineered attenuated vaccine candidates. A third aim has been the characterization of genetic diversity among wild-type HAV isolates using PCR-related technology, as this will facilitate clinical studies of HAV vaccines and future military disease surveillance activities.

INTRODUCTION

Hepatitis A as a military hazard Compared with the military forces of many other nations, American soldiers, marines and sailers have a relatively low prevalence of antbody to hepatitis A virus (HAV) and are thus particularly susceptible to hepatitis A, a potentially debilitating infectious disease which may reach epidemic proportions under poor sanitary conditions. Due to substantial improvements in public health sanitation within the United States over the past five decades, the prevalence of HAV has continued an overall decline within most regions of the country. As a result, American forces personnel demonstrate an extremely low prevalence of antibodies to the virus (Lemon and Bancroft, 1983). Among American civilian populations, overseas travel to HAV endemic regions represents a substantial risk factor for acquisition of hepatitis A. This risk is substantially magnified when Americans traveling overseas are military forces deployed to developing regions. Even greater risks may be anticipated when previously existing public health facilities and sanitation practices have been disrupted by military conflict, such as occurred within the Kuwaiti Theater of Operations. The large scale mobilization of the military forces of the United States during Operations Desert Shield and Desert Storm reemphasized the risks and special problems posed by HAV to American military operations. While short-term protection against hepatitis A may be provided by passive administration of immune globulin (IG), supplies of IG may be strained during massive mobilizations such as Desert Shield. Moreover, readministration of IG is required at 6 months intervals in order to maintain continued protection and this may be particularly difficult to accomplish with troops engaged in action against hostile forces. There is thus an urgent need for development of a vaccine capable of providing safe, long-term, active immunity against HAV, and which would be available to the military forces of the United States at reasonable cost.

Commercial interest in development of formalin-inactivated HAV vaccines has resulted in products that are safe and immunogenic (for a recent review, see Siegl and Lemon, 1990). Recent clinical studies during the past 12 months have demonstrated clinical efficacy in children following administration of one or two doses of commercially prepared formalin-inactivated HAV vaccine (Both Merck and Smith-Kline vaccines). However, these vaccines are likely to require multiple administrations in order to elicit even moderately long-lasting protective levels of immunity. Multiple-dose schedules, with late booster doses given at 6 months after the first immunization may prove inconvenient for use in military populations. Perhaps of greater practical significance, the cost of these vaccines is likely to be high. This is due to the comparatively poor in vitro yields of antigen obtained with current vaccine virus strains, and the purification procedures required for production of an acceptable, modern vaccine. The Smith-Kline vaccine is currently sold in Switzerland at a cost of SF 39.00 (approximately US$ 27.00) per dose. Such high costs prohibit the universal use of inactivated vaccines among U.S. military forces. Because of the uncertainties concerning future use of inactivated HAV vaccines, work under this grant focuses on efforts to develop alternative approaches to development of HAV vaccines.

Synthetic immunogens for protection against HAV In previous work, we mapped an immunodominant neutralization antigenic site on the surface of the HAV capsid by analysis of murine monoclonal antibody-resistant neutralization escape variants of HAV (Ping et al., 1988; Cox et al., 1990), and by characterizing the competition between such monoclonal antibodies for binding to the virus capsid (Stapleton et al., 1987). These studies indicate that the B- C loops of capsid proteins VP3 and VP1 contribute to an immunogenic structure on the virus surface that dominates in the human immune response (Ping et al., 1988; Day et al., 1990a). Although, this site is largely conformationally defined, we have reasoned that short oligopeptide sequences representative of the relevant regions

of VP3 and VP1 should be both antigenic and potentially immunogenic with respect to HAV neutralizing activity. This has been shown to be the case with peptides representative of antigenic sites in other picornaviruses, including type 1 poliovirus (Chow et al., 1985), human rhinovirus 14 (Francis et al., 1989), and foot-and-mouth disease virus (FMDV) (Bittle et al., 1982). Peptide immunogens are highly stable reagents; they are potentially very inexpensive and extremely safe inasmuch as they are chemically defined. We have previously shown that only low levels of neutralizing antibody are required for protection against hepatitis A (Stapleton et al., 1985), a fact which was recently confirmed during an efficacy trial with the Merck inactivated HAV vaccine. We have thus postulated that peptide immunogens may have practical application to the prevention of hepatitis A.

We have taken several experimental approaches to this problem:

(1) We have continued efforts to map the neutralization epitopes of HAV by the isolation and characterization of monoclonal antibody resistant neutralization escape HAV mutants. We have developed significant new information concerning the antigenic structure of the HAV capsid utilizing this approach, and have now partially mapped epitopes recognized by most of a large panel of murine and human monoclonal antibodies (see Ping and Lemon, 1992).

(2) We have synthesized octapeptides representing the primary sequence of the HAV capsid proteins VP3 and VP1 on polyethylene pins, and probed such peptides with monoclonal and polyclonal anti-HAV antibodies in peptide ELISAs (PEPSCAN) (Geysen et al., 1984, 1987). During the past year, this approach has identified a linear antigenic determinant within the HAV capsid structure which is recognized by a neutralizing murine monoclonal antibody. Peptides representing this linear determinant have been synthesized, and are now being assessed for the ability to elicit a virus neutralizing response in small animals.

(3) We have characterized the antigenicity and immunogenicity of chimeric picornaviruses in which appropriate HAV peptide sequences have replaced residues within an antigenic loop of capsid protein VP1 of the Sabin type 1 poliovirus. These antigenic chimeras have been constructed using an infectious poliovirus cDNA clone which contains a mutagenesis cassette in the region encoding for VP1 (Burke et al., 1989). HAV/poliovirus chimeras have the potential of presenting HAV peptides in a conformationally constrained manner, and we have been encouraged by preliminary success with such chimeric viruses (see Progress Report Nos. 1 and 2, and Lemon et al., 1992).

Improved candidate attenuated HAV vaccines In addition to exploring these approaches to subunit HAV vaccines, we have continued efforts to develop an infectious HAV cDNA clone derived from a highly cell culture-adapted virus (p16 HM175). This work follows that supported under a previous contract with the U.S. Army Medical Research and Development Command (DAMD17-85-C-5272). Commercial attempts to develop an attenuated HAV vaccine have focused on the attenuation of HAV that accompanies adaptation of virus to growth in cell culture (for a review, see Lemon, 1985; Siegl and Lemon, 1990). In general, such cell culture-adapted viruses appear to replicate poorly in the primate liver, and have very poor immunogenicity in man. There is thus a need less highly passaged cell culture-adapted variants, or to develop novel approaches to selecting attenuated HAV vaccine candidate strains.

In an effort to characterize mutations which are responsible for the cell culture-adaptation and attenuation of HAV, we previously molecularly cloned and fully sequenced a cell culture-adapted HAV variant (HM175/p16 virus) which was initially isolated by Binn

and coworkers at the Walter Reed Army Institute of Research. Although owl monkey challenge experiments with a passage-related virus, HM175/S30 (a neutralization escape variant) suggested that HM175/p16 might be virulent, an ongoing study being conducted in collaboration with COL. James LeDuc, USAMRIID, and Dr. Leonard Binn, WRAIR, suggests that the plaque-purified HM175/p16 virus may be highly attenuated in owl monkeys. If so, this recent finding attaches additional significance to the mutations we had previously identified in the HM175/p16 virus. These mutations include only a single nonsilent mutation in protein 2C and two nonsilent mutations in 2B, proteins which have been suggested by Emerson et al. (1991) to contribute to the growth properties in cell culture and attenuation phenotype of the NIH attenuated HM175 vaccine candidate.

We have demonstrated that specific mutations present within the 5' nontranslated region (5'NTR) of HM175/p16, at bases 152 and/or 203-7 and base 687, act to enhance the growth of HAV in BS-C-1 cells. The growth enhancing effects of these mutations are restricted to BS-C-1 cells and are not present in FRhK-4 cells which are derived from an alternative simian species. In related work, we have identified the secondary structure elements of the 5' NTR of the HAV genome within which these mutations are located (Brown et al., 1991). We have shown that the mutation at 687 is within a functional ribosomal landing pad in the 5'NTR, which controls the initiation of HAV translation. These mutations are also close to the RNA-binding sites for a 39 kDa cellular protein (p39) which we have recently shown binds specifically to regions of the HAV 5'NTR and which may well represent a mammalian translation initiation factor which functions in internal ribosomal entry. This work has provided valuable new information concerning the structural and functional organization of the 5'NTR, and suggests new approaches by which the genome could be manipulated to create additional candidate attenuated viruses. Specifically, we have shown that the HAV 5' NTR structure is very similar to that of the murine cardioviruses. Because cardioviruses with deletion mutations within the poly-C tract of the cardioviral 5' NTR may be highly attenuated in vivo, yet grow well in cell culture, (Duke et al., 1990), we suspect that HAV mutants with deletions within the first pyrimidine-rich tract (bases 95-152) may have interesting attenuation properties. We are currently preparing such mutants.

We previously constructed a genomic-length cDNA clone derived from HM175/p16 (Jansen et al., 1988) within the transcriptional vector pGEM-3. The full-length HM175/p16 cDNA clone was not viable in transfection assays, either as RNA or DNA, but transfection experiments with chimeric molecules constructed with an existing infectious full-length clone (pHAV/7, Cohen et al., 1987) suggested that a lethal mutation existed between bases 4977 and 7003 of the p16 clone (see Progress Report No. 1). Complete sequencing of the p16 construct demonstrated a lethal, frame shifting mutation within this region. This defect has been corrected, but infectivity of the new construct has not yet been documented. The recent demonstration that HM175/p16 may be substantially attenuated lends added significance to this work. Additional transfections are in progress.

Genetic variation among HAV strains Finally, we reported in years 1 and 2 the development of a simplified polymerase chain reaction (PCR)-based method for analysis of the nucleotide sequence of wild-type HAV isolates, and its application to the characterization of wild-type HAV strains. During the past year, we collaborated with Dr. Betty Jo Robertson of the Viral Hepatitis Branch of the Centers for Disease Control, Atlanta, GA, and Yasuo Moritsugu of the National Institute of Health, Tokyo, Japan, in evaluating the genotype of over 150 unique HAV isolates and antigen-positive fecal samples collected from all regions of the world. These strains comprise the vast majority of all HAV strains available worldwide. This effort has provided new information concerning the genetic diversity of HAV and the molecular epidemiology of the virus (Robertson et al., 1992), which will be useful to future military and civilian disease control efforts.

RESEARCH PROGRESS

1. Neutralization epitopes of HAV

Rationale X-ray crystallographic determinations of virus structures have contributed substantially to current understanding of the structural organization and function of picornaviruses (Hogle et al, 1985; Rossmann et al, 1985; Luo et al, 1987; Acharya et al, 1989). Combined with the analysis of neutralization escape mutants selected for resistance to monoclonal antibodies, structural studies have provided a uniquely detailed view of the antigenic features of these viruses (Page et al, 1988; Rossmann et al, 1985; Boege et al, 1991). However, technical difficulties have severely hampered such studies with HAV, which is now classified as the type species of the genus *Hepatovirus* within the family *Picornaviridae* (Francki et al, 1991). The replication cycle of HAV in cell culture is relatively slow and yields of virus are considerably lower than those obtained with most other picornaviruses (Binn et al, 1984). Thus the production of the quantities of purified virus that are required for crystallographic studies represents a daunting task. In addition, HAV replication is usually nonlytic and neutralization of HAV *in vitro* often relatively inefficient (Lemon et al, 1985), making the isolation and characterization of neutralization escape mutants both tedious and difficult.

Nonetheless, some information concerning the antigenic structure of HAV has been obtained from studies with a very limited number of escape mutants. As reported in previous Annual Reports, we previously isolated a small series of escape mutants by repetitive cycles of neutralization and amplification of virus in the presence of murine monoclonal antibodies (Stapleton et al, 1987; Ping et al, 1988). Neutralization studies with these escape mutants and a related panel of monoclonal antibodies suggested that most murine antibodies recognize a dominant antigenic site on the virus capsid. These results were further supported by studies examining competition between various monoclonal antibodies for binding to the surface of the virus capsid (Hughes et al, 1984; Stapleton et al, 1987; Ping et al, 1988). Sequencing studies indicated that the Asp-70 residue of capsid protein VP-3 (Asp 3-070*) plays a critical role in forming this antigenic site, while a lesser contribution is made by Ser 1-102 (Ping et al, 1988). These residues are well conserved among human HAV strains, consistent with the highly conserved antigenic characteristics of this virus. Subsequent mutagenesis studies with infectious cDNA, have confirmed the importance of Asp 3-070 in the antigenic structure of HAV (Cox et al, 1991).

During the past year, we characterized an expanded series of HAV escape mutants that resist neutralization by one or more murine monoclonal antibodies. Results of these studies are consistent with a critical role for Asp 3-070 in the structure of an immunodominant antigenic site, but also document the contribution of additional residues of VP1 to this domain and suggest the existence of a second, potentially independent antigenic site involving other residues of VP1. These results are of interest because they shed additional light on the structure of this virus, and thus allow further comparisons of HAV with other picornaviruses. However, a better understanding of the antigenic structure of HAV is also critically relevant to the rational design of new candidate HAV vaccines.

Selection of neutralization escape mutants Attempts to neutralize HAV almost always result in substantial nonspecific nonneutralized fractions (Lemon et al, 1985), even

*We use a four-digit nomenclature to describe specific amino acid residues: y-nnn, in which "y" represents the capsid protein (VP1, VP2, or VP3), and "xxx" the residue number from the proposed amino terminus (Cohen et al, 1987).

after vigorous attempts to eliminate virus aggregation. Thus, we subjected virus to repeated cycles of neutralization followed by amplification in the presence of murine monoclonal antibodies, using a general approach that we have described previously (Stapleton et al, 1987). However, to facilitate the process of mutant selection, we utilized an epitope-specific radioimmunofocus assay technique (Lemon et al, 1990; Lemon et al, 1991) following the first 3-4 cycles of neutralization and virus amplification. To reduce aggregation, an HM175/18f virus harvest (cell culture supernatant fluids) was brought to 0.1% sodium dodecyl sulfate (SDS) and held at 37 °C for 30 min, followed by extraction with an equal volume of chloroform and sonication for 3 mins. An aliquot containing approximately 10^7 radioimmunofocus-forming units (RFU) was neutralized by incubation with a monoclonal antibody (2D2, AD2, AE8, or H7C27) overnight at 4 °C followed by 1 hr at 35.5 °C, prior to adsorbtion to nearly confluent monolayers of BS-C-1 cells in replicate 25 cm^2 tissue culture flasks (3 or 6 flasks, labelled A, B, C, etc.) for 2 hr at 35.5 °C. The concentration of each monoclonal antibody was 10- to 1000-fold greater than that required to acheive 50% neutralization of virus. The inoculum was removed and the cell sheet was washed three times with E-MEM containing 2% fetal bovine serum. The cells were refed with 5 ml maintenance medium containing 1/10th the original concentration of monoclonal antibody, and incubated at 35.5 °C. After 7 days incubation, the medium was removed and used as a source of virus for the second cycle of neutralization/amplification. This medium was extracted once with an equal volume of choroform prior to the addition of fresh antibody for the subsequent neutralization cycle.

After 3-4 cycles of neutralization/amplification, the neutralization resistance of virus present in cell culture supernatant fluids was assessed in an epitope-specific radioimmunofocus assay (Lemon et al, 1990). This virus was treated with SDS and neutralized with monoclonal antibody as described above, and 10-fold dilutions of neutralized virus were inoculated onto nearly confluent BS-C-1 cell monolayers in 60 mm polystyrene petri dishes. After 2 hr virus adsorbtion, the cell sheets were overlaid with medium containing 0.5% Seakem agarose. Following 7 days incubation at 35.5 °C in a 5% CO_2 atmosphere, the overlay was carefully removed and placed at 4 °C, the cells fixed with 80% acetone, and stained with the cognate ^{125}I-labelled monoclonal antibody (Lemon et al, 1983). Following autoradiographic exposure, the cell sheet was counterstained with ^{125}I-labelled polyclonal human convalescent antibody (JC) to HAV and a second autoradiographic exposure was obtained. This epitope-specific radioimmunofocus assay procedure allowed the overall neutralization-resistance of the virus harvest to be assessed by comparison of virus titers before and after neutralization. However, the double-antibody staining procedure also identified individual virus clones which no longer bound the monoclonal antibody of interest. At least two monoclonal antibody-resistant clones were recovered from each original neutralization/amplification culture series (A, B, C, etc.), by removal of agarose plugs overlying selected radioimmunofoci. These agarose plugs were processed for virus recovery as described previously (Lemon et al, 1985). When the epitope-specific radioimmunofocus assay results indicated that the virus harvest was a mixture of resistant and non-resistant viruses, the resistant (mutant) clones were plaque-purifed twice by this procedure. Otherwise, mutant viruses were only plaque-purified once prior to amplification. Virus recovered from overlays was neutralized overnight with the cognate antibody and amplified in 25 cm^2 flasks in the presence of the monoclonal antibody, to prepare a virus seed for determination of neutralization resistance to the complete panel of antibodies and for sequencing of virion RNA. If no escape mutants were identified in the epitope-specific radioimmunofocus assay, the virus was subjected to an additional 3 neutralization/amplification cycles, as described above.

Analysis of neutralization-resistance phenotypes Escape mutant harvests were prepared from supernatant fluids of 25 cm^2 cell culture flasks, 7 days after inoculation of virus, and the virus titer determined by radioimmunofocus assay. A standard inoculum of

each mutant virus (approximately 400 RFU/ml), prepared in medium containing 2% fetal bovine serum, was mixed with an equal volume of antibody for neutralization, as described above, and the residual virus titer determined by radioimmunofocus assay (Lemon et al, 1983). We defined neutralization resistance as <30% neutralization, partial neutralization resistance as 30-60% neutralization, and neutralization sensitivity as > 60% neutralization. For comparison, a parental HM175/18f virus control was included for each monoclonal antibody tested in each neutralization assay.

Nucleotide sequence of escape mutants The partial nucleotide sequence of clonally isolated escape mutants was determined by dideoxynucleotide sequence analysis of the products of an antigen capture/polymerase chain reaction method described previously (Jansen et al, 1990). Polyclonal human antibody (JC) was utilized for capture of virus. Positive (+) and negative (-) strand oligodeoxynucleotide primer pairs utilized for reverse transcription and polymerase chain reaction amplification of cDNA included: a) +560/-1501, b) +1561/-1771, c) +2646/-3192, d) +1561/-2037, e) +2392/-2698, f) +1308/-1771, g) +1998/-2556, and h) +1308/-2556, where the number indicates the position of the most 5' base within the positive strand represented by the oligonucleotide (wild-type HM175 numbering)(Cohen et al, 1987). PCR amplification products were subjected to agarose gel electrophoresis, the specific band excised and extracted with phenol/chloroform. Multiple positive-strand and negative-strand primers were utilized for ^{35}S dideoxynucleotide sequencing of the double-stranded cDNA.

Characterization of murine monoclonal antibodies to HAV We asssembled a panel of 24 murine monoclonal antibodies, raised against 8 different strains of human HAV in 9 different laboratories. Four of these antibodies were of the IgM isotype, 1 was IgA, and the remaining 19 antibodies were IgG (Table 1). Fifteen IgG antibodies were of the subclass IgG_{2a}, while 2 antibodies were IgG_3, and 1 each IgG_{2b} and IgG_1. Fourteen of 15 antibodies for which the light chain type was determined were of the κ type. Serial dilutions of monoclonal antibodies were tested for their ability to block the binding of polyclonal human convalescent antibody to HAV in a solid-phase radioimmunoassay (Table 1). Eighteen of these antibodies were capable of blocking polyclonal antibody binding $\geq$ 50%. In addition, we characterized each antibody with respect to its ability to compete against two individual reference monoclonal anti-HAV antibodies (K34C8 and B5B3) for binding to HAV in solid-phase immunoassays. We previously demonstrated that these two antibodies do not complete with each other for binding to the virus capsid, and that a combination of these two antibodies is capable of blocking the binding of polyclonal human antibody almost completely (Stapleton et al, 1987; Ping et al, 1988). All but two antibodies (1.134 and LSH-14H) had substantial neutralization activity against HM175/18f virus ($\geq$ 85% reduction in infectious virus titer) (Table 1). The two monoclonal antibody preparations with low neutralizing activity (<50% neutralization), one of which was IgM(κ) and the other IgG_{2a}, were excluded from further analysis.

Isolation of neutralization escape mutants We have shown previously that HM175 virus mutants with His or Ala substitutions at Asp 3-070 resist neutralization by many murine monoclonal antibodies (Ping et al, 1988). Additionally, an escape mutant having a Leu substitution at Ser 1-102 (mutant S32) demonstrated partial resistance to several antibodies. As mutations at either of these residues confer resistance to several antibodies (2D2, B5B3 and 3E1), these data suggested that residues 3-070 and 1-102 both contribute to a single, dominant neutralization site on the capsid surface (Ping et al, 1988). To test this hypothesis, we determined whether multiple, independently selected mutants capable of escaping neutralization by the monoclonal antibody 2D2 would demonstrate mutations in both VP3 and VP1.

The isolation of escape mutants resistant to 2D2 required between 3 and 6 neutralization/amplification cycles, as indicated in Table 2. Mutant viruses isolated from each of six independent neutralization/amplification culture series failed to bind 2D2 in an epitope-specific radioimmunofocus assay (data not shown), but each was detected with radiolabelled polyclonal human antibody. Two mutants were clonally isolated from each of the 6 culture series (A through F). Both clones from 5 of the 6 culture series had identical mutations within the nucleotide sequence (and were thus considered to be sibling clones), while the two mutants from the remaining culture series (series B) each had unique mutations. Thus, we isolated a total of 7 independent mutants that were resistant to 2D2. Each of these seven mutants had closely spaced mutations within VP3 (Table 2). Four mutants had an Asn substitution at Asp 3-070, while two had a Tyr substitution at the same residue and one an Arg substitution at Gln 3-074. As we have confirmed previously the involvement of residue 3-070 in the dominant antigenic site of HAV, we carried out only limited sequencing of these VP3 mutants. Thus, the selection of multiple virus variants which were resistant to 2D2 resulted in the identification of only a very narrow range of residues (3-070 and 3-074) at which responsible mutations were found.

We selected three other antibodies (AD2, AE8 and H7C27) for generation of additional escape mutants, because of evidence that these antibodies are directed against epitopes that are distinct from that bound by 2D2. Two of these antibodies have monoclonal antibody competition profiles in solid-phase radioimmunoassays that are substantially different from that of 2D2 (Ping and Lemon, 1992). The competition profile of the third antibody, H7C27, is relatively unique in that it has approximately equal blocking activity against K34C8 and B5B3, but is unable to block the binding of either more than about 70% (Ping and Lemon, 1992). In addition, while resistance to AD2 was conferred by a His substitution at Asp 3-070 (mutant S30), AE8 and H7C27 were able to neutralize previously isolated 3-070 and 1-102 mutants.

For both AD2 and AE8, we selected independent mutants from three parallel neutralization/amplification culture series. These escape mutants generally required a higher number of neutralization cycles for isolation than escape mutants selected against 2D2, as resistant virus was identified in only 1 of 6 culture series after 4 neutralization/amplification cycles (series AE8-A), compared with 6 of 7 culture series carried in the presence of 2D2 (Table 2). While the neutralization titer of the AD2 ascitic fluid (50% neutralization endpoint between 10^{-3} and 10^{-4}) was significantly lower than that of 2D2 (between 10^{-5} and 10^{-6}), this was not the case with AE8 (also between 10^{-5} and 10^{-6}). Thus the greater number of neutralization/amplification cycles required with AE8 and AD2 could only be partially related to a lower level of neutralizing activity of these antibodies. In addition to their lack of neutralization ($\leq$ 10%) with the cognate antibody, the AD2 and AE8 mutants failed to bind this antibody in epitope-specific radioimmunofocus assays (data not shown).

Two mutant viruses were clonally isolated from each neutralization/ amplification culture series. Both clonal variants isolated from each of the 3 culture series selected for AE8 resistance had a Glu substitution at Val 1-171 (Table 2). The same substitution was also present in both clonal isolates from one culture series selected for AD2 resistance (series AD2-A), while an Asp substitution at Ala 1-176 was found in both clonal isolates from each of the other two AD2 neutralization/amplifcation culture series (Table 2). As mutations at 1-171 and 1-176 of HAV have not been associated previously with neutralization escape, we determined the complete P1 sequence of two representative mutants, 11C (Glu 1-171 selected against AE8) and 20D (Asp 1-176 selected against AD2) (Fig. 1). In both cases, no other nonsilent mutations from the HM175/18f parent nucleotide sequence were identified within the P1 region. These results thus indicate that AD2 and AE8 recognize closely spaced epitopes involving residues 171 and 176 of VP1.

We also selected mutants against antibody H7C27 in three separate neutralization/amplification culture series. In each case, escape mutants which failed to bind H7C27 in an epitope-specific radioimmunofocus assay were present after 4 cycles of neutralization/amplification (Table 2). Two escape mutants were clonally isolated from each of the 3 culture series for further analysis. We found that each of these escape mutants had undergone mutation within the Lys 1-221 codon (Table 2). Both clonal isolates from culture series A had a Glu 1-221 substitution, due to a base change at position 2868 (HM175 wild-type numbering), while the 2 mutants from culture series B had a Met 1-221 substitution due to a base change at position 2869. On the other hand, while both mutants (33M and 24B) isolated from culture series C had Asn 1-221 substitutions, these were due to different base changes at the third codon position, 2870 (Table 2). Thus, four unique H7C27-resistant mutants were isolated, each with amino acid replacements at residue 1-221. As mutations at this residue have not been associated previously with neutralization escape, we determined the complete P1 nucleotide sequence of mutant 33M (Asn 1-221). We found no other nonsilent mutation from the nucleotide sequence of the HM175/18f parent (Fig. 1).

Cross-resistance of escape mutants to other monoclonal antibodies We characterized the neutralization-resistance phenotype of escape mutants by testing each for resistance to each of the 22 monoclonal antibodies which we found to have significant neutralizing activity against the parent HM175/18f virus (Table 1). For these studies, we included mutant viruses with unique amino acid substitutions, selecting only one representative mutant when multiple mutants with the same amino acid replacement had been isolated. These results are summarized in Fig. 2, in addition to the neutralization-resistance phenotypes of three previously isolated mutants, S32, S30 and 43c (Ping et al, 1988). Neutralization resistance was determined in radioimmunofocus reduction assays, using concentrations of antibody which were capable of effectively neutralizing an HM175/18f virus inoculum in parallel assays.

Mutants with amino acid substitutions at Asp 3-070 demonstrated partial or complete neutralization resistance against 16 of the 22 antibodies (Fig. 2). In addition, although mutant S32 (Leu 1-102, selected against B5-B3) was sensitive to meutralization by K24F2 according to the stringent criteria for neutralization resistance employed for this study (< 60% neutralization), we have previously shown in replicate experiments that S32 is less susceptible to neutralization by this antibody than is its parent, HM175/p16 virus (Ping et al, 1988). The broadest resistance was observed with mutants which had either His or Ala replacements at residue 3-070 (previously isolated mutants S30 and 43c), while mutant 7A which has an Arg substitution at Gln 3-074 had a considerably narrower resistance profile (Fig. 2). These results indicate that residues Asp 3-070 and Gln 3-074 contribute to an important neutralizing antigenic domain which is dominant in the murine antibody response.

Mutants 20D and 11C, which were selected against antibodies AD2 and AE8 respectively, were also resistant to H80C25, 7E7, and H29C26 (partial resistance only) (Fig. 2). As resistance to AD2 and H80C25 was also conferred by a His substitution at Asp 3-070, these results indicate that Val 1-171 and Ala 1-176 (sites of mutations in 11C and 20D) contribute to this same immunodominant antigenic site. This was further confirmed by the resistance pattern of mutant S32. This mutant has a Leu replacement at Ser 1-102 (Ping et al, 1988) and was partially resistant to antibodies 2D2, 3E1, and H80C25, as well as B5B3 against which it was originally selected (Stapleton et al, 1987). Thus the dominant antigenic site of HAV includes contributions from Asp 3-070, Gln 3-074, Ser 1-102, Val 1-171 and Ala 1-176. Twenty of 22 murine neutralizing antibodies may be related to this site by resistance due to mutations at one or more of these residues (Fig. 2). This site is complex, however, and contains multiple epitopes, as resistance to most individual

antibodies was mediated by mutations within only one or two putative protein loops on the surface of the capsid. Antibody H80C25 represents an exception to this statement, however, as partial or complete resistance was mediated by substitutions within the three putative protein loops containing residues 3-070, 1-102, 1-171, or 1-176.

All 3 escape mutants with unique substitutions at Lys 1-221 were resistant only to antibody H7C27, the antibody against which they had been selected (Fig. 2). Conversely, this antibody effectively neutralized all of the remaining escape mutants. Thus, the available data suggest that the antigenic site identified by mutations at Lys 1-221 may be functionally distinct from the immunodominant site described above. Similarly, antibody 4E7 neutralized each of the escape mutants, including those with mutations at Lys 1-221 (Fig. 2). Thus the antigenic site against which 4E7 is directed remains undefined, and may possibly represent yet a third, functionally independent site.

2. PEPSCAN analysis of HAV capsid proteins

This approach to mapping antigenic peptides of HAV involves the synthesis of nested octapeptides on polyethylene pins. These peptides, overlapping each other by 7 residues, are probed in enzyme-linked immunosorbent assays (ELISA) which assess the binding of immunoglobulins to specific peptide-bearing pins (PEPSCAN) (Geysen et al., 1984, 1987) (see previous Annual Reports). In previous years, we screened pin-based octapeptides representative of the putative B-C loops of VP3 (residues 3-050 - 3-091) and VP1 (residues 1-080 - 1-130) of HAV against the panel of neutralizing anti-HAV monoclonal antibodies described above. No reactivity was demonstrated with these monoclonal antibodies, even though analysis of escape mutants (see above) indicates that some of these antibodies recognize epitopes which are comprised, in part, of amino acid residues 3-070, 3-074 or 1-102. Thus, these epitopes are highly conformational and do not contain linear, antigenic peptides.

During the past year, we extended the PEPSCAN analysis to include the region from 1-131 to 1-300, as we have shown that this region contains two additional domains involved in epitopes recognized by neutralizing monoclonal antibodies. We found that the murine monoclonal H7C27 specifically and reproducibly binds a set of nested octapeptides representing a functional linear antigenic site near the carboxy terminus of VP1 of HAV (Fig. 3). These data are the first firm evidence of a linear antigenic site in the HAV capsid, and reasonably raise our expectations that synthetic peptides representing this domain may be immunogenic and capable of eliciting neutralizing antibody responses. We have thus synthesized three peptides in bulk which represent this putative antigenic site. These peptides have been conjugated to keyhole limpet hemocyanin and administered to series of guinea pigs and rabbits in collaboration with Dr. John Cullen of the College of Veteinary Medicine of North Carolina State University.

It is interesting to note that the antibody bound by this linear epitope, H7C27, is unique in that neutralization escape mutants raised to H7C27 only escape mutation by this and no other antibody (Fig. 2) (Ping and Lemon, 1992). These mutants have undergone replacement of the amino acid residue at 1-221. However, the H7C27 epitope identified by PEPSCAN is located approximately 30 residues further toward the carboxy terminus of VP1. These data suggest that mutations at 1-221 are not located within the primary antibody recognition site, but rather affect the conformation of the protein loop identified by PEPSCAN (or otherwise make this loop non-available for antibody binding).

3. Analysis of HAV/poliovirus chimeras

Previous studies concerning HAV/poliovirus chimeras were published during the past year (see Lemon et al., 1992). At present, we are planning construction of additional chimeras containing the H7C27 linear epitope that we have demonstrated by PEPSCAN (see previous section). For this purpose, we have received the pCAS7 infectious poliovirus clone from Dr. Jeffrey Almond of the University of Reading. This clone contains a mutagenesis cassette within the region encoding the B-C loop of VP1, which constitutes antigenic site 1 of Sabin strain poliovirus type 1. This plasmid thus allows rapid replacement of the B-C loop of the poliovirus vaccine strain with the putative HAV antigenic determinant. We will assess the antigenicity and immunogenicity of chimeric viruses rescued from the recombinant clone.

4. Role of 5' NTR mutations in adapation of HAV to growth in cell culture

Passage of human HAV in cell culture results in progressive increases in the efficiency with which the virus replicates in cell culture. Because the presence of identical mutations within the 5' nontranslated regions (5'NTRs) of several independent cell culture-adapted HAV variants suggests that the 5'NTR may play a role in determining this change in virus host range, we constructed chimeric infectious cDNA clones in which regions of the 5'NTR of cell culture-adapted HM175/p35 virus were replaced with cDNA from either wild-type virus (HM175/wt) or a second independently isolated, but closely related cell culture-adapted virus (HM175/p16). Substitution of the complete 5'NTR of HM175/p35 with the 5'NTR of HM175/wt resulted in virus with very small replication foci in continuous monkey kidney (BS-C-1) cells, indicating that 5'NTR mutations in HM175/p35 virus are important for growth in these cells. A chimera with the 5'NTR sequence of HM175/p16 retained the large focus phenotype of HM175/p35 virus, indicating that mutations present in HM175/p16 could substitute for those present in HM175/p35. The growth properties of other viruses having chimeric 5'NTR sequences indicated that mutations at bases 152 and/or 203-7 are required for efficient replication in BS-C-1 cells, while a mutation at base 687 of HM175/p16 has a minor role in enhancing growth. A comparison of the antigen content and size of replication foci suggested that these 5'NTR mutations enhance the spread of virus among BS-C-1 cells, but do not influence the amount of viral antigen expressed within individual cells. In contrast to BS-C-1 cells, these 5'NTR mutations did not enhance replication in FRhK-4 cells, which are derived from a different simian species (Fig. 4). Thus, mutations at bases 152 and/or 203-7 of the 5'NTR enhance the replication of HAV in a highly host cell-specific fashion. Because recent data suggests that HM175/p16, like HM175/p35 virus, may be substantially attenuated in otherwise susceptible primates, these data raise the distinct possibility that these 5'NTR mutations contribute to the attneuation phenotype.

5. Structure and Function of the 5' NTR of HAV

As described in Annual Report No. 2, we carried out a phylogenetic analysis of sequence covariance within the 5'NTRs of 9 unique human and simian HAV strains (see also Brown et al., 1991). We manually identified covariant nucleotide substitutions predictive of conserved helical structures, and utilized this information to constrain the folding of the 5'NTR in thermodynamic predictions of secondary structure made by the RNAFOLD (Wisconsin Software Package) and STAR (Abrahams et al., 1990) programs. The resulting model was subsequently tested and refined by nuclease digestion experiments in which we determined the effects of single- and double-strand specific ribonucleases on synthetic RNA in the region between bases 280-735. This work led us to propose the structure shown in Annual Report No. 2 for the 5'NTR of HAV (see also, Brown et al., 1991).

Based on preliminary studies involving the in vitro translation of 5' terminally truncated RNA transcripts representing segments of the 5'NTR (Brown et al., 1991), we considered it likely that the NTR contains an internal ribosomal entry site (IRES) functioning in the initiation of viral translation (see Annual Report No. 2). To formally prove the existence of an HAV IRES, we made a series of bicistronic constructs containing two reporter genes separated by the 5'NTR. These constructions were made as a single transcriptional unit under the control of the T7 promoter in the vector pGEM3/zf-. The T7 terminator sequence of pGEMEX-2 (Promega) was placed downstream of the second reporter gene (the HAV capsid precusor VP0-ΔVP3) to facilitate eventual use of these plasmids in the BT7-6 cellular expression system (see below). The upstream reporter gene encoded the small form of hepatitis delta virus antigen ($p24^{\delta}$) (small BamHI/PstI fragment of pX9, a gift from Michael Lai). The $p24^{\delta}$ gene is unique in that it contains two translational stop codons (codons 196 and 215) which are both functional in vivo and in vitro (Chao et al., 1991). Any read-through at the first stop codon would lead to expression of the large form of delta antigen ($p27^{\delta}$), which would be readily detectable in gels. In several additional constructions, the 5'NTR segment placed between the two reporter genes was progressively deleted from the 5' end. In the absence of RNA degradation, leaky ribosomal scanning, termination/reinitiation, or internal ribosomal entry, there should be no translation of the second reporter gene when RNA transcripts made from these constructs are used to program translation in vitro or in vivo.

Translation of pSD transcripts (containing only the $p24^{\delta}$ gene) in RRL resulted only in expression of $p24^{\delta}$ (Fig. 5). The absence of $p27^{\delta}$ indicated complete termination of translation at the first stop codon. However, when the HAV 5'NTR and P1 coding region were placed downstream of the $p24^{\delta}$ gene, there was abundant expression of the HAV polyprotein. Expression of the HAV polyprotein was dependent upon the presence of an intact 5'NTR, confirming the existence of an IRES. HAV translation was markedly reduced when bases 1-354 of the NTR were removed (pSD-NTRΔ355, Fig. 5), indicating that the 5' limit of the IRES extends upstream of base 355. Minimal detectable translation continued even with deletion to base 634 (pSD-NTRΔ634). This might reflect retention of a minimal level of IRES activity with the 634-735 segment. That it is not due to leaky scanning of the upstream cistron is suggested by the total elimination of HAV translation with deletion to residue 740 (pSD-Δ740). This deletion leaves intact the second in-frame AUG of the HAV polyprotein (AUG-12, which is codon 3, the prefered translational start codon as recently shown by Tesar et al. 1992), but does alter bases at the -2, -3, and -4 positions. The identity of delta and HAV translation products was confirmed by immunoprecipitation with specific anti-peptide antibodies (data not shown). These results confirm the existence of an IRES extending from upstream of base 355 to base 735 (the first in-frame AUG).

6. Mutations associated with cytopathic HAV

We reported in Annual Report No. 1 that HAV variants recovered from persistently infected green monkey kidney (BS-C-1) cells (pHM175 virus) induced a cytopathic effect during serial passage in BS-C-1 or fetal rhesus kidney (FRhK-4) cells. Epitope-specific radioimmunofocus assays showed that this virus comprised two virion populations, one with altered antigenicity including neutralization-resistance to monoclonal antibody K24F2, and the other with normal antigenic characteristics (Lemon et al., 1991). We demonstrated that replication of the antigenic variant was favored over virus with normal antigenic phenotype during persistent infection, while virus with the normal antigenic phenotype was selected during serial passage. Viruses of each type were clonally isolated; both were cytopathic in cell cultures and displayed a rapid replication phenotype when compared to the noncytopathic HM175/P16 virus which was used to establish the original persistent infection. The clonal variant which has normal antigenic phenotype (HM175/18f) has

exceptional in vitro growth properties, and was supplied to Dr. Leonard Binn of the Department of Virology, Walter Reed Army Institute of Research for use in HAV neutralization assays done in support of HAV vaccine trials sponsored by WRAIR.

As described in Annual Report No. 2, we determined the nearly complete genomic sequences of the two cytopathic virus clones HM175/43c (the antigenic variant) and HM175/18f. The genomes of these viruses were found to contain 30 and 33 nucleotide changes from the sequence of HM175/P16 respectively (Lemon et al., 1991). Both viruses shared a common 5' sequence (bases 30-1677), as well as sequence identity in the P2/3 region (bases 3249-5303 and 6462-6781) and 3' terminus (bases 7272-7478). VP3, VP1 and $3C^{pro}$ contained different mutations in the two virus clones, with amino acid substitutions at residues 70 of VP3 and 197 and 276 of VP1 of the antigenic variant. These capsid mutations did not affect virion thermal stability. A comparison of the nearly complete genomic sequences of three clonally isolated cytopathic variants was suggestive of genetic recombination between these viruses during persistent infection, and indicated that mutations in both 5' and 3' nontranslated regions and in the nonstructural proteins 2A, 2B, 2C, 3A, and $3D^{pol}$ may be related to the cytopathic phenotype.

During the past year, we constructed a series of recombinant full-length infectious cDNA clones in which we replaced the 5'NTR, P2 and 3'NTR regions of the infectious pHAV/7 clone with the corresponding cDNA amplified from HM175/18f virion RNA by polymerase chain reaction. These validity of these constructions was confirmed by DNA sequence analysis. Transfection of FRhK-4 cells with RNA transcribed from these constructs has shown that mutations in the P2 region of HM175/18f determine the rapid replication (RR+) phenotype of this virus (generating large foci in 7 days compared with 14 days in radioimmunofocus assays) (Fig. 6). These results were subsequently confirmed in BS-C-1 cells using virus rescued from the original transfections. Surprisingly, there was no apparent enhancement of growth of pHAV/7 (HM175/p35) recombinants in either cell type with the inclusion of only the 5'NTR of HM175/18f virus. Given the mutations in HM175/18f defined by us previously (see Annual Report No. 2), these results suggest a primary role for the 2C gene of HM175/18f virus in controlling the enhanced growth of this virus in cell culture. These findings open new avenues to the construction of viruses and cell culture systems having enhanced capacity for production of HAV antigen. We are currently defining in greater detail the role played by individual P2 proteins in determining the RR+ phenotype.

7. Molecular Epidemiology and Genetic Diversity of HAV

In collaboration with Dr. Betty Robertson of the Hepatitis Branch, Centers for Disease Control and Dr. Yasuo Moritsugu of the NIH, Japan, we have established an extensive data base containing the partial genomic sequences of over 150 unique HAV strains. Collectively, we have determined 168 bases of sequence from each virus at the putative VP1/2A junction, using the AC/PCR method (Jansen et al., 1990). Development of this method was described in detail in the final annual report of our former contract with the U.S. Army Research and Development Command (DAMD17-85-C-5272) and in Report No. 1 of this grant submitted in 1990. This analysis of partial genomic sequences has confirmed the existence of considerable genetic diversity among human HAV strains (Jansen et al., 1990). These findings are summarized in Figure 7 and are now in press (Robertson et al., 1992). Results of these studies support the existence of 7 distinct HAV genotypes (defined arbitrarily as strains differing at more than 15% of bases sequenced), four of which are human viruses and three of which appear to be restricted to primates (African green monkey and the cynomolgus monkey). A number of human isolates have been identified which are related closely in sequence to the PA21 strain which was previously isolated from New World owl monkeys held at the Gorgas Memorial Laboratory

in Panama by Binn and Lemon at the Walter Reed Army Institute of Research. The current evidence thus suggests that PA21 is representative of genetically distinct human HAV strains (genotype III), and is not in fact a simian agent. Thus far, there is no evidence for significant antigenic variability among human viruses from different genotypes, although the possibility of such genetic variability remains as detailed studies of antigenicity have thus far only been carried out with genotype I and II strains (Lemon et al., 1992).

CONCLUSIONS

During the past year, we completed the characterization of HAV neutralization escape mutants that were selected for resistance to the murine monoclonal antibodies 2D2, AE8, AD7 and H7C27. Cross-neutralization studies involving a panel of 12 unique mutants with a panel of 22 neutralizing monoclonal antibodies have confirmed the existence of an immunodominant neutralization site, with two functionally distinct but overlapping domains, and a second, apparently totally independent antigenic site involving residue 1-221. PEPSCAN analysis indicates that the immunodominant HAV antigenic site is highly conformational, but that the 1-221 site (H7C27 epitope) contains a linear antigenic domain that is recognized in solid-phase peptide ELISA assays. The actual H7C27 antibody-binding site appears to be located a short distance from the site of the H7C27 escape mutations at 1-221, suggesting that these mutations in someway destroy the conformation of this site or otherwise sequester it from antibody. Synthetic peptides have been made which represent the linear H7C27 epitope, and are now being assessed for their ability to induce neutralizing antibodies to HAV. In addition, we will construct an HAV/poliovirus chimera, by inserting this linear HAV determinant into antigenic site I of the Sabin type 1 poliovirus. These experiments will be facilitated by use of an infectious cDNA clone of Sabin type 1 virus which contains a mutagenesis cassette within antigenic site 1. The immunogenicity and antigenicity of the chimeric virus will be studied during the next year. We will continue efforts to isolate escape mutants selected for resistance to 4E7 (a murine antibody which neutralizes all previously isolated mutants and thus might be directed against a unique, yet unrecognized antigenic site), and human monoclonal antibodies to HAV (to determine whether these recognize epitopes which are identical to those recognized by the murine antibodies). Sequencing of the P1 region of the RNA from these new mutants should provide additional information concerning the location of a possible third minor antigenic site, as well as define potential differences in the human and murine antibody response to the virus.

Recent studies have characterized the genetic determinants of the rapid replication (RR+) phenotype of the cytopathic HAV variant, HM175/18f virus, and have shown that the P2 region is of primary importance in defining the RR+ phenotype. This information will be of use in engineering virus variants with enhanced growth for use in vaccine production or diagnostic assays. We have recently constructed a stably-transfected cell line from permissive BS-C-1 cells which constituitively expresses the T7 bacteriophage RNA polymerase (Day and Lemon, unpublished data). We will use this cell line to express P2 proteins of the cytopathic HM175/18f strain in *trans*, and we will determine the impact of these proteins on replication of RR- HAV strains in these permissive cells. Despite this evidence that the P2 proteins play an important role in cell culture adaptation, our analysis of viruses containing chimeric 5' NTRs which are derived in part from wild-type and attenuated HAVs has indicated an important role for the 5'NTR in controlling replication in cell culture (Day et al., 1992). We have shown that the 5'NTR is closely related in structure to the 5' NTR of the murine cardioviruses, and quite distinct from the structure of the poliovirus 5' NTR. We have shown that the HAV 5'NTR contains a functional IRES element, and that mutations which enhance growth in cell culture are located within

important structural elements of the IRES. We suspect that viable mutants with deletions within the 5'NTR will have interesting attenuation phenotypes. Such mutants are currently under construction.

The partial genomic sequencing of over 150 HAV strains using the AC/PCR approach will be useful in analysis of HAV strains isolated during future HAV vaccine trials, and ir future military disease surveillance activities. These studies now include almost all isolated of HAV available world-wide.

LITERATURE CITED

Acharya R, Fry E, Stuart D, Fox G, Rowlands D, Brown F. 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 A resolution. Nature 337:709-716.

Binn LN, Lemon SM, Marchwicki RH, Redfield RR, Gates NL, Bancroft WH. 1984. Primary isolation and serial passage of hepatitis A virus strains in primate cell cultures. J. Clin. Microbiol. 20:28-33.

Bittle JL, Houghten RA, Alexander H, Shinnick TM, Sutcliffe JG, Lerner RA, Rowlands DJ, Brown F. 1982. Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature 298:30-33.

Boege U, Kobasa D, Onodera S, Parks GD, Palmenberg AC, Scraba DG. 1991. Characterization of mengo virus neutralization epitopes. Virology. 181:1-13.

Brown E, Jansen RW, Lemon SM. 1989. Antigenic and genetic analysis of a simian strain of hepatitis A virus (HAV): comparison with human HAV. J Virol 63:4932-4937.

Brown EA, Day SP, Jansen RW, Lemon SM. 1991. The 5' nontranslated region of hepatitis A virus: secondary structure and elements required for translation in vitro. J. Virol. 65:5828-5838.

Burke KL, Evans DJ, Jenkins O, Meredith J, D'Sousa EDA, Almond J. 1989. A cassette vector for the construction of antigen chimeras of poliovirus. J Gen Virol 70:2475-2479.

Chow M, Yabrov R, Bittle JL, Hogle JM, Baltimore D. 1985. Synthetic peptides from four separate regions of the poliovirus type 1 capsid protein VP1 induce neutralizing antibodies. Proc Natl Acad Sci USA 82:910-914.

Cohen JI, Ticehurst JR, Purcell RH, Buckler-White A, Baroudy BM. 1987. Complete nucleotide sequence of wild-type hepatitis A virus. comparison with different strains of hepatitis A virus and other picornaviruses. J. Virol. 61:50-59.

Cohen, J.I., J.R. Ticehurst, S.M. Feinstone, B. Rosenblum, and R.H. Purcell. 1987. Hepatitis A virus cDNA and its RNA transcripts are infectious in cell culture. J. Virol. 61:3035-3039.

Cox EM, Emerson SU, Lemon SM, Ping L-H, Stapleton JT, Feinstone SM. 1991. Use of oligonucleotide-directed mutagenesis to define the immunodominant neutralization antigenic site of HAV. In Brown F, Chanock RM, Ginsberg HS, Lerner RA eds. "Vaccines 90. Modern Approaches to New Vaccines Including Prevention of AIDS." Cold Spring Harbor. Cold Spring Harbor Laboratory Press, pp. 169-173.

Day S, Lemon SM. 1990. A single base mutation in the 5' nontranslated region of HAV enhances replication in vitro. In: Brown F, Chanock RM, Ginsberg HS, Lerner RA, eds. Vaccines 90, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 175-178.

Day S, Brown EA, Jansen RW, Lemon SM. 1992. Mutations in the 5' nontranslated region of hepatitis A virus RNA which enhance growth in cell culture. Submitted.

Francki RIB, Fauquet CM, Knudson DL, Brown F. 1991. Classification and Nomenclature of Viruses Arch. Virol./Suppl. 2. Wien. Springer-Verlag,

Geysen HM, Meloen RH, Barteling SJ. 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc Natl Acad Sci USA 81:3998-4002.

Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoofs PG. 1987. Strategies for epitope analysis using peptide synthesis. J Immunol Methods 102:259-274.

Hogle JM, Chow M, Filman DJ. 1985. Three-dimensional structure of poliovirus at 2.9 A resolution. Science 229:1358-1365.

Hughes JV, Stanton LW, Tomassini JE, Long WJ, Scolnick EM. 1984. Neutralizing monoclonal antibodies to hepatitis A virus. partial localization of a neutralizing antigenic site. J. Virol. 52:465-473.

Jansen RW, Newbold JE, Lemon SM. 1988. Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for *in vitro* replication. Virology 163:299-307.

Jansen RW, Siegl G, Lemon SM. 1990a. Molecular epidemiology of hepatitis A virus defined by an antigen-capture/polymerase chain reaction method. Proc Natl Acad Sci USA 87:2867-2871.

Lemon SM, Binn LN, Marchwicki RH. 1983. Radioimmunofocus assay for quantitation of hepatitis A virus in cell cultures. J. Clin. Microbiol. 17:834-839.

Lemon SM, Binn LN. 1983. Serum neutralizing antibody response to hepatitis A virus. The J. Infect. Dis. 148:1033-1039.

Lemon SM, Binn LN. 1985. Incomplete neutralization of hepatitis A virus *in vitro* due to lipid-associated virions. J. Gen. Virol. 66:2501-2505.

Lemon SM, Jansen RW. 1985. A simple method for clonal selection of hepatitis A virus based on recovery of virus from radioimmunofocus overlays. J. Virol. Methods 11:171-176.

Lemon SM. 1985. Type A viral hepatitis: new developments in an old disease. N. Engl. J. Med. 313:1059-1067.

Lemon, S.M., L.N. Binn, R. Marchwicki, P.C. Murphy, L.-H. Ping, R.W. Jansen, L.V.S. Asher, J.T. Stapleton, D.G. Taylor, and J.W. LeDuc. 1990. In vivo replication and reversion to wild-type of a neutralization-resistant variant of hepatitis A virus. J. Infect. Dis. 161:7-13.

Lemon, S.M., P.C. Murphy, P.A. Shields, L.-H. Ping, S.M. Feinstone, T. Cromeans, and R.W. Jansen. 1991. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. J. Virol. 65:2056-2065.

Lemon SM, Barclay W, Ferguson M, Murphy P, Jing L, Burke K, Wood D, Katrak K, Sangar D, Minor PD, Almond JW. 1992. Immunogenicity and antigenicity of chimeric picornaviruses which express hepatitis A virus (HAV) peptide sequences:

Evidence for a neutralization domain near the amino terminus of VP1 of HAV. Virology. In press.

Luo M, Vriend G, Kamer G, Minor I, Arnold E, Rossmann MG, Boege U, Scraba DG, Duke GM, Palmenberg AC. 1987. The atomic structure of Mengo virus at 3.0 A resolution. Science 235:182-191.

Page GS, Mosser AG, Hogle JM, Filman DJ, Rueckert RR, Chow M. 1988. Three-dimensional structure of poliovirus serotype. 1 neutralizing determinants. J. Virol. 62:1781-1794.

Palmenberg, A.C. 1989. Sequence alignments of picornaviral capsid proteins, p.211-241. In B. Semler and E. Ehrenfeld (eds.), Molecular Aspects of Picornavirus Infection and Detection. ASM Press, Washington, DC.

Ping L-H, Jansen RW, Stapleton JT, Cohen JI, Lemon SM. 1988. Identification of an immunodominant antigenic site involving the capsid protein VP3 of hepatitis A virus. Proc Natl Acad Sci USA 85:8281-8285.

Ping L-H, Lemon SM. 1992. Hepatitis A virus neutralization epitopes defined by analysis of neutralization escape mutants selected against murine monoclonal antibodies. J. Virol. In press.

Rossmann MG, Arnold E, Erickson JW, Frankenberger EA, Griffith JP, Hecht H-J, Johnson JE, Kamer G, Luo M, Mosser AG, Rueckert RR, Sherry B, Vriend G. 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature 317:145-153.

Siegl G, Lemon SM 1990. Recent advances in hepatitis A vaccines. Virus Res. 17:75-92.

Stapleton JT, Jansen RW, Lemon SM. 1985. Neutralizing antibody to hepatitis A virus in immune serum globulin and in the sera of human recipients of immune serum globulin. Gastroenterology 89:637-642.

Stapleton JT, Lemon SM. 1987. Neutralization escape mutants define a dominant immunogenic neutralization site on hepatitis A virus. J. Virol. 61:491-498.

APPENDIX

Table 1. Murine monoclonal antibodies to hepatitis A virus.

Antibody	Ig Class	Virus Strain	Source[1]	Competition vs. pcAb[2]	Neutralization[3]
K34C8	$IgG_{2a}(\kappa)$	HM790	g	%	92 %
1.193	$IgA(\kappa)$	HM175	h	74.9	98
K24F2	IgG_{2a}	HM790	g	59.7	100
H10C29	Ig_{G3}	GBM	b	39.7	91
K32F2	IgG_{2a}	HM790	g		100
813	$IgG_3(\kappa)$	CF53	a		97
141C19	$IgG_{2a}(\kappa)$	GBM	b	60.9	99
6A5	IgG_{2a}	CR326	e		91
AG3	$IgG_{2a}(\kappa)$	S841	f	69.9	100
1009	$IgG_1(\kappa)$	CF53	a		100
H14C42	$IgM(\lambda)$	GBM	b	49.9	96
1B9	IgG_{2a}	CR326	e		99
2D2	IgG_{2a}	CR326	e	64.5	94
3E1	IgG_{2a}	CR326	e		86
B5B3	IgG_{2a}	KMW1	i		98
H80C25	$IgG_{2a}(\kappa)$	GBM	b	64.7	85
AD2	$IgG_{2a}(\kappa)$	S841	f	86.5	95
7E7	$IgG_{2a}(\kappa)$	GBM	c	76.5	96
AE8	$IgM(\kappa)$	S841	f	66.1	92
H29C26	$IgG_{2a}(\kappa)$	GBM	b	71.0	97
H7C27	$IgG_{2b}(\kappa)$	GBM	b	70.2	94
4E7	$IgM(\kappa)$	GBM	c	73.3	97
1.134	$IgM(\kappa)$	HM175	h	13.3	<50
LSH-14H	IgG_{2a}	LSH/S	d	36.0	<50

[1]Sources:

a. D. Crevat, Clonatec, Paris, France
b. R. Decker, Abbott Laboratories, N. Chicago, IL
c. B. Flehmig, University of Tubingen, Tubingen, Germany
d. H. Garelick, London School of Tropical Medicine and Hygiene, London, United Kingdom
e. J. Hughes, Merck Sharp and Dohme Research Laboratories, West Point, PA
f. C. Li, Sichuan Health and Anti-Epidemic Station, China
g. Commonwealth Serum Laboratories, Melbourne, Australia
h. LH. Ping, University of North Carolina, Chapel Hill, NC
i. R. Tedder, Middlesex Hospital, London, United Kingdom

[2]Maximum percent competition (at highest antibody concentration) against polyclonal human antibody (JC) in a solid-phase radioimmunoassay employing HM175/18f antigen.

[3]Percent neutralization HM175/18f virus at working concentration.

Antibody	Mutant	Series	Cycle	Base Change	Asp 3-070	Gln 3-074	Ser 1-102	Val 1-171	Ala 1-176	Lys 1-221
K24F2	S30	A	3	1677	His					
B5B3	S32	A	3	2512			Leu			
-	43c	-	-	1678*	Ala					
-	30M	-	-	1677	Asn					
2D2	27A	A	6	1677	Asn					
	18A	B	3	1677	Asn					
	3A	B	4	1677	Tyr					
	7A	C	4	1690		Arg				
	22A	D	3	1677	Asn					
	28A	E	3	1677	Tyr					
	34B	F	3	1677	Asn					
AD2	4C	A	6	2719				Glu		
	16C	B	6	2734					Asp	
	20D	C	6	2734					Asp	
AE8	40A	A	4	2719				Glu		
	11C	B	6	2719				Glu		
	47M2	C	6	2719				Glu		
H7C27	15C	A	4	2868						Glu
	20B	B	4	2869						Met
	33M	C	4	2870						Asn
	24B	C	4	2870						Asn

*also at 2797 and 3033

Table 2. Neutralization escape mutants derived from the HM175 strain of HAV

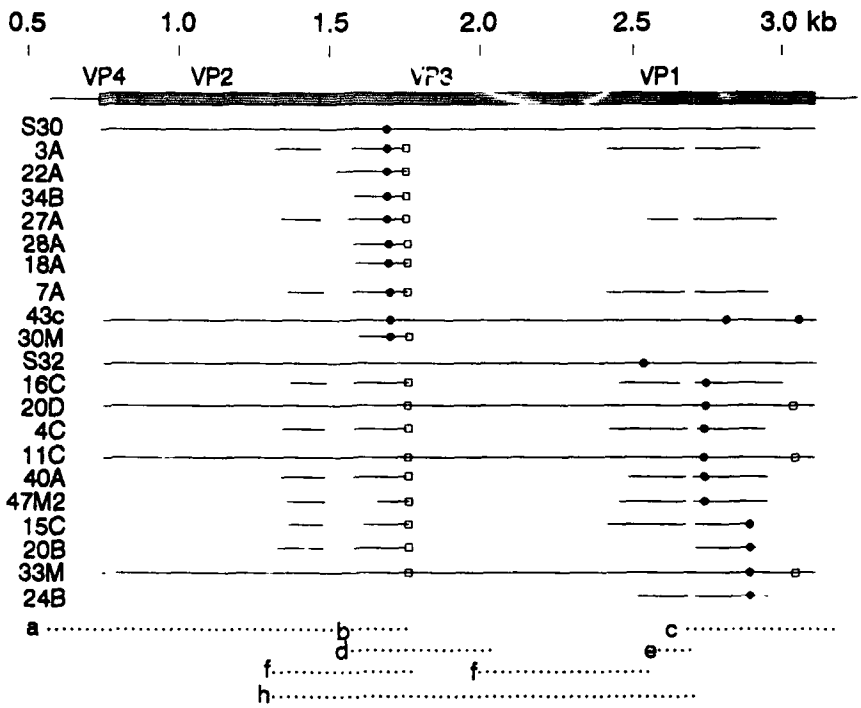

Figure 1. Genomic regions sequenced within individual neutralization escape mutants (listed at the left). The map of the P1 region of the HM175 virus genome is shown at the top, with the location of bases encoding each capsid protein indicated. Horizontal lines indicate regions for which the nucleotide sequence was determined. The complete P1 sequence of mutants S30, S32, 43c, 20D, 11C, and 33M has been determined.(•) represent non-silent mutations within the nucleotide sequence, while (o) indicate non-silent mutations (from the wild-type virus) present in the parent HM175/18f virus from which all mutants other than S30, S32, and 43c were derived. Silent mutations are not shown. The location of cDNA segments amplified by PCR for sequence determination is shown at the bottom.

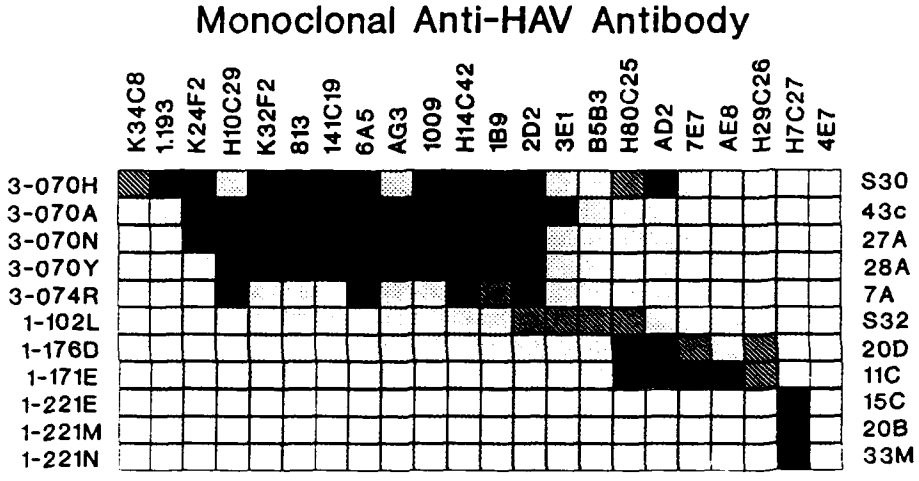

Figure 2. Cross-resistance of neutralization escape mutants to a panel of 22 neutralizing murine monoclonal antibodies. Eleven mutants (listed to the right), representing unique amino acid substitutions within the capsid proteins (listed to the left) were characterized as resistant (<30% neutralization, solid boxes), partially resistant (30-60% neutralization, striped boxes), or sensitive (>60% neutralization, open boxes) to each antibody.

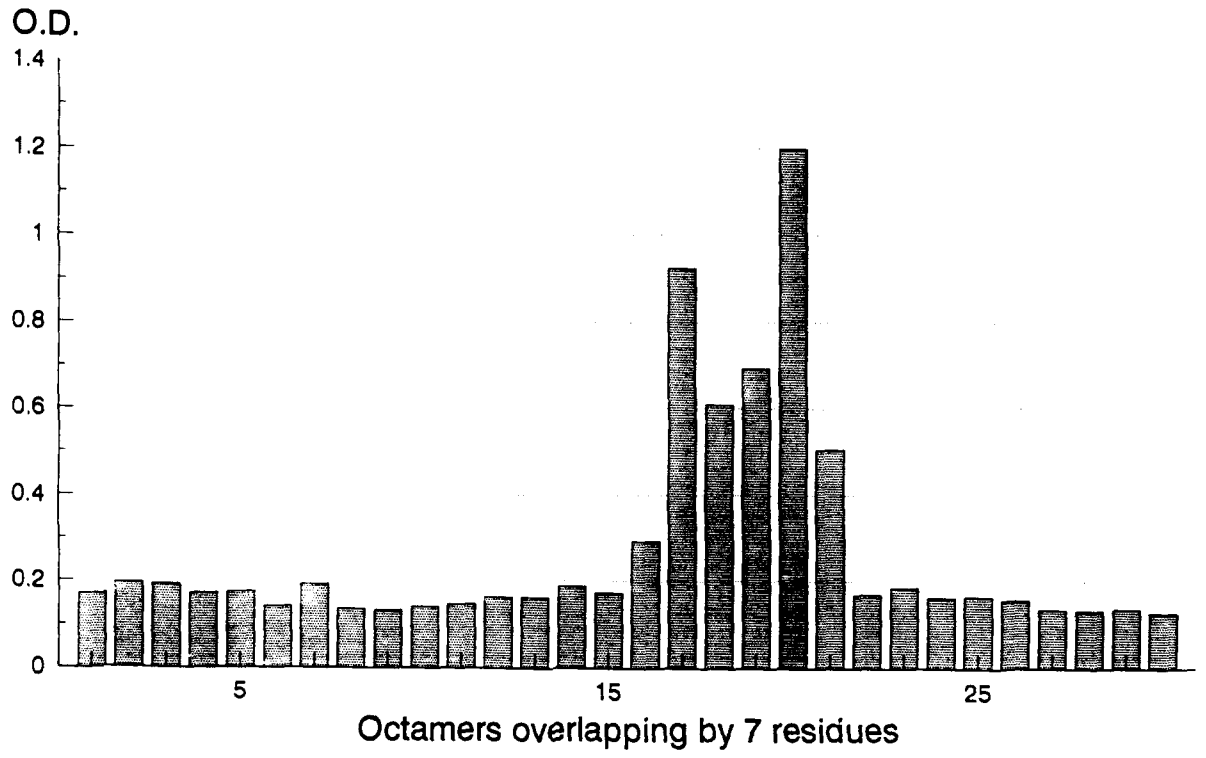

Figure 3. Peptide ELISA (PEPSCAN) demonstrating reactivity of the neutralizing murine monoclonal antibody H7C27 with a series of nested octapeptides (adjacent peptides overlapping each other by 7 residues) representing a linear antigenic domain near residue 221 of VP1.

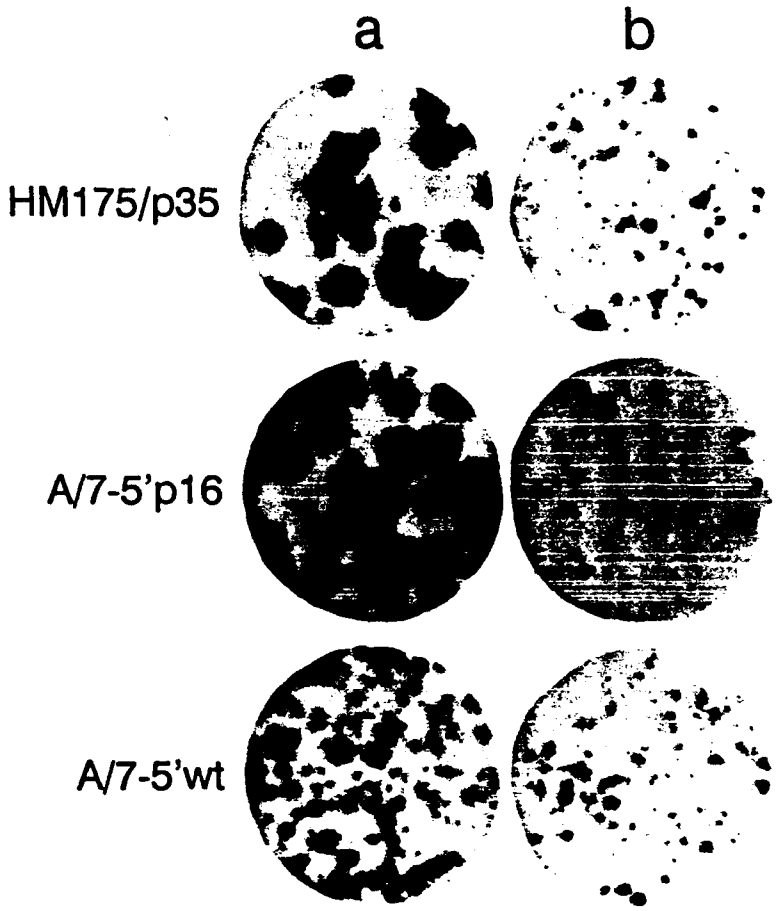

Figure 4. Radioimmunofoci generated by virus rescued from plasmids pG3HAV/7, pA/7-5'p16 and pA/7-5'wt. Assays were carried out in continuous African green monkey (BS-C-1) (column "a") or fetal rhesus monkey (FRhK-4) (column "b") cells. pG3HAV/7 contains the full-length sequence of HM175/p35 virus (pHAV/7). pA/7-5'p16 is identical to pG3HAV/7 with the exception that the 5'NTR has been replaced by corresponding the HM175/p16 sequence. In pA/7-5'wt, the 5'NTR sequence is that of wild-type HM175 virus. See Annual Report No. 2.

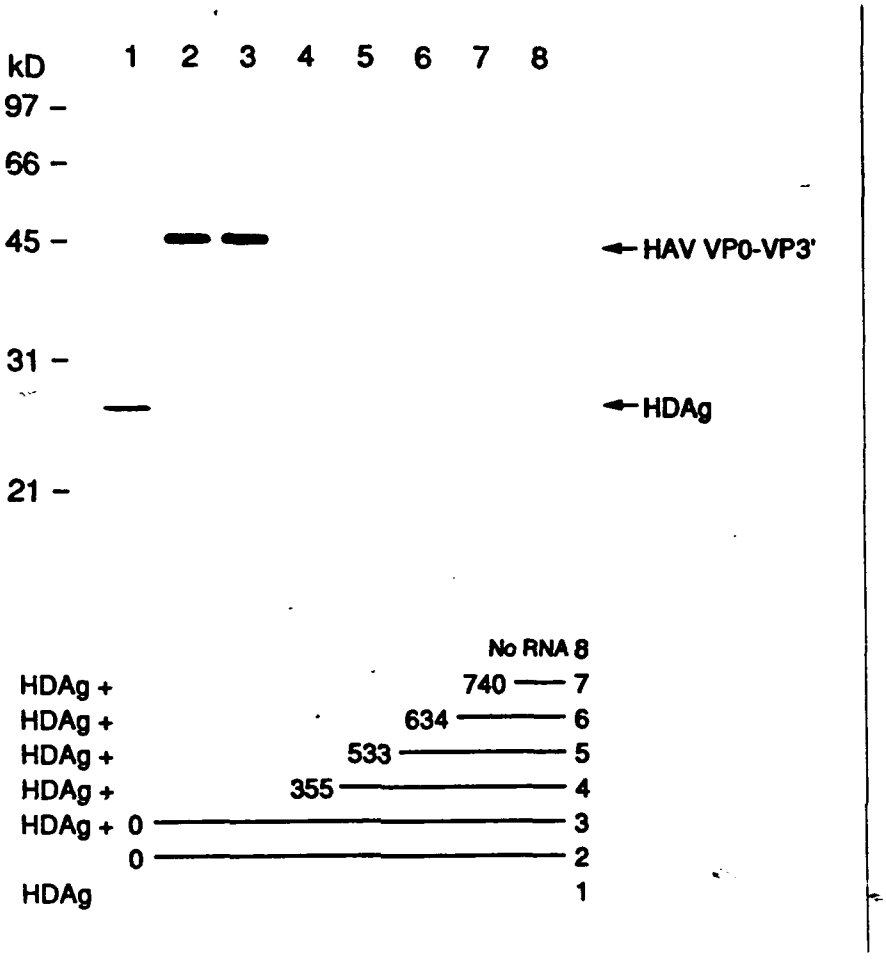

Figure 5. SDS-PAGE of translation products produced in rabbit reticulocyte lysates programmed with bicistronic RNA transcripts in which the upstream reading frame encodes the small hepatitis delta antigen ($p24^{\delta}$), and the downstream reading frame encodes a truncated HAV P1 polyprotein (VP0-ΔVP3). The two reading frames are separated by the 5'NTR of HAV, or partial 5'NTR segments that are 5' terminally truncated as indicated. Control translations of monocistronic transcripts ($p24^{\delta}$ or VP0-ΔVP3) are also shown.

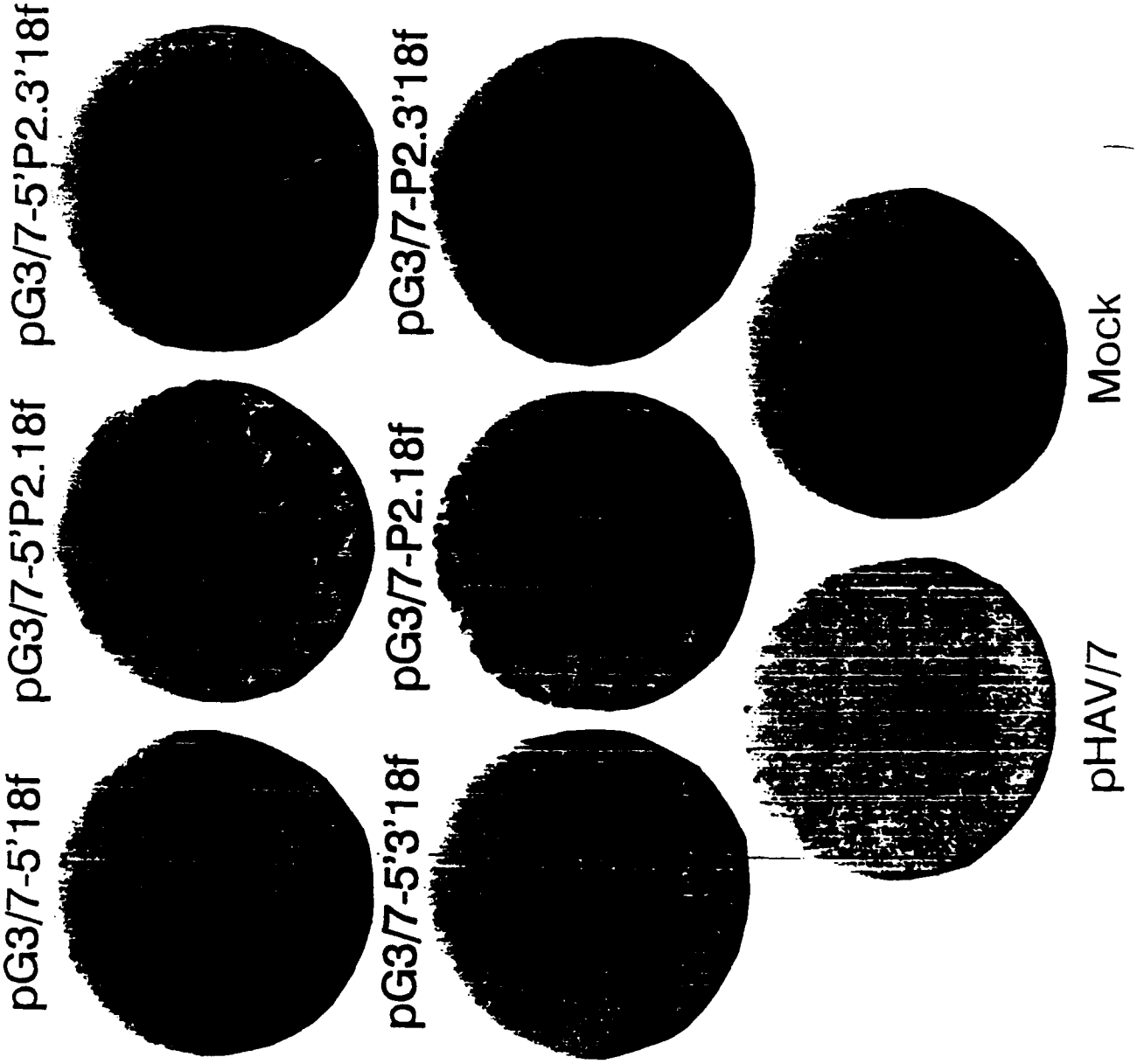

Figure 6. Radioimmunofocus RNA transfection assay demonstrating enhanced growth of virus in cells transfected with pG3/7-P2.18f (pHAV/7 containing P2 genomic region of HM175/18f), compared with pHAV/7 (HM175/p35 virus). Cells were processed for antigen detection at 7 days.